PRODUCTS AND METHODS FOR GAUCHER DISEASE THERAPY

FIELD OF THE INVENTION

The invention relates to products and methods for medical treatment of Gaucher disease and, in particular, nucleic acid molecules, polypeptides and vectors for polypeptide or gene therapy treatment.

BACKGROUND OF THE INVENTION

Gaucher disease is a lysosomal storage disease caused by the deficiency of functional glucocerebrosidase (Gcc) enzyme. Gcc is present in all cell types. The defective enzyme cannot break down a fatty substance, glucocerebroside, which is an important component of cell membranes. The fat accumulates in macrophages (which are known as the "Gaucher cells"). The fat-laden macrophages are found typically in the liver, spleen, bone marrow and lungs. The amount of the enzyme deficiency varies from person to person as do the symptoms. Some patients may show no clinical symptoms, while others may die from the disease. The symptoms of the disease and mutant forms of Gcc that cause Gaucher disease are described, for example, in U.S. 5,266,459 (Beutler) and U.S. 5,234,811 (Beutler and Sorge).

There are therapies for Gaucher disease. Ceredase is a form of the Gcc enzyme from placenta that is able to metabolize the fat in Gaucher cells. The enzyme restores normal function to a Gaucher cell. The amount of enzyme used in treatment varies. As much as 30-60 units per kilogram of bodyweight (U/kg/bw) may be given every other week. Positive results have been reported with 2.3 U/kg/bw given three times a week. Lower doses, such as 1-5 U/kg/bw twice weekly, have also been used with success, but this is less frequent. The intarcellular half life of the enzyme is up to 60 hours. A large number of placentas are needed to make sufficient Ceredase, so this form of therapy is very expensive. It has been almost completely replaced by treatment with a recombinant form of the enzyme, Cerezyme but this therapy is also expensive. Cerezyme is dispensed as a powder whereas Ceredase comes as a liquid. Sterile water must be added to the Cerezyme bottle to dissolve the powder. The shelf life of the drugs is short (<3 months), and splitting doses is cumbersome and wasteful. Allergic reactions to Ceredase are common, but rarely life-threatening. Adverse reactions to Cerezyme appear to be less common, but experience with the drug is still very limited.

Gcc has been structurally modified in order to obtain improved pharmacokinetics over naturally occurring Gcc (which is derived from placenta). These modifications include amino acid modifications as well as carbohydrate changes. For example, U.S. 5,549,892 discloses a

recombinant polypeptide that differs from naturally occurring Gcc by the presence of histidine in place of arginine at position 495. In another embodiment, the carbohydrate remodeled recombinant Gcc has increased fucose and N-acetyl glucosamine residues compared to remodeled naturally occurring Gcc. The increased pharmacokinetics of these compounds provides a therapeutic effect at doses that are lower than those required using remodeled, naturally occurring Gcc. However, this Gcc remains expensive to provide. Furthermore, improved pharmacokinetics does not necessarily compensate for inadequate bioavailability of Gcc.

Gene therapy has been administered to Gaucher patients. All experiments carried out to date have been undertaken using ex vivo, retrovirus-mediated transfection, which requires sophisticated laboratory facilities and is very expensive. Although transgene expression could be demonstrated in mice undergoing this procedure, experiments in humans have been disappointing. No clinically significant Gcc gene expression has been reported in humans undergoing retrovirus-mediated transfection with existing Gcc gene preparations. One problem of gene therapy is in reproducibly obtaining high-level, tissue-specific and enduring expression from genes transferred into cells. Currently, there is no suitable gene therapy vector that expresses at a high level for Gaucher disease gene therapy.

SUMMARY OF THE INVENTION

The invention includes a modified Gcc cDNA insert that can be inserted into any mammalian expression vector for use in the medical treatment of Gaucher disease. In a preferred embodiment, the modified cDNA was inserted into a vector named pINEX2.0 which was then used to transfect mammalian cells. When pINEX2.0 containing the unmodified Gcc cDNA coding sequence, pINEX5'GCC3', was transfected into cells, their RNA purified from cell lysates and subjected to reverse transcription followed by the polymerase chain reaction (RT-PCR), two distinct major bands were observed after agarose gel electrophoresis (Fig. 1). Isolation, purification and sequencing of the RT-PCR products identified a major aberrantly spliced mRNA species which encodes only a 19 amino acid peptide before encountering a STOP codon. Surprisingly, this aberrant splicing event occurred completely within the Gcc cDNA coding sequence (Fig. 2), *i.e.* no vector sequences were involved. Site directed mutagenesis was performed to modify the nucleotide sequence in the region of aberrant mRNA splicing without affecting polypeptide coding (Fig. 3). Modifications were aimed at disrupting the known consensus sequences for RNA-splicing (Krawczak et al. 1992). The effectiveness of these modifications were tested by transient transfection into CHO cells, followed by our human-

specific immunoprecipitation assay for Gcc. Data (n=18) indicate a 5±1 (Std. Error)-fold increase in Gcc activity was achieved when the modified replaced the unmodified insert in the pINEX2.0 expression vector.

The invention relates to an isolated Gcc DNA molecule, wherein the DNA molecule has a modification in at least one nucleotide that disrupts a splicing consensus sequence and prevents splicing of mRNA produced from the DNA molecule, while preserving the ability of the DNA to express active Gcc. The modification impairs a consensus nucleotide sequence needed to induce splicing. The DNA molecule is preferably modified at two cryptic splice sites. The DNA preferably includes a mutation in the 3' junction site. In one embodiment, the mutation is as shown in the 3' junction site in Table 1, or a functionally equivalent mutation. In another embodiment, the DNA molecule includes a mutation in the 5' splice junction site. The mutation is preferably as shown in the 5' junction site in Table 1, or a functionally equivalent mutation.

The DNA molecule preferably includes all or part of the nucleotide sequence shown in figure 4(b).

Another aspect of the invention relates to a vector including a DNA molecule of the invention. The vector preferably includes a promoter that is functional in a mammalian cell.

The invention also includes mRNA produced from the DNA molecule or vector of the invention.

Another aspect of the invention relates to a method of medical treatment of Gaucher disease in a mammal, including administering to the mammal an effective amount of a nucleic acid molecule of the invention or a vector of the invention and expressing an effective amount of the polypeptide encoded by the nucleic acid molecule for alleviating clinical symptoms of Gaucher disease.

The invention includes a host cell, or progeny thereof, including a nucleic acid molecule of the invention. The host cell is preferably selected from the group consisting of a mammalian cell, a human cell and a Chinese Hamster Ovary cell. The invention also includes a method for producing a recombinant host cell capable of expressing a Gcc nucleic acid molecule, the method including introducing into the host cell a vector of the invention. The invention also includes a method for expressing a Gcc polypeptide in a host cell including culturing the host cell under conditions suitable for DNA molecule expression. Another aspect of the invention relates to a method for producing a transgenic cell that expresses elevated levels of Gcc

polypeptide relative to a non-transgenic cell, including transforming a cell with a vector of the invention.

The invention includes an isolated polypeptide encoded by and/or produced from a nucleic acid molecule of th invention, or a vector of the invention.

The invention includes a method of producing a genetically transformed cell which expresses or overexpresses a Gcc polypeptide, including: a) preparing a Gcc nucleic acid molecule according to any of claims 1-18; b) inserting the nucleic acid molecule in a vector so that the nucleic acid molecule is operably linked to a promoter; c) inserting the vector into a cell. The invention includes a transgenic cell produced according to the method of the invention.

The invention also includes a pharmaceutical composition, including a carrier and (i) a nucleic acid molecule of the invention (ii) a vector of the invention or (iii) Gcc polypeptide produced from (i) or (ii), in an effective amount for reducing clinical symptoms of Gaucher disease. The carrier preferably carrier includes a liposome.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention will be described in relation to the drawings in which:

Figure 1. Separation of RT-PCR products from CHO cell permanently transfected with pINEX-5'-GCC-3'.

Figure 2. Diagrammatic Representation of possible RT-PCR products representing mRNA splice variants from pINEX-5'-GCC-3'.

Figure 3. Comparison of consensus splice site donor/acceptor site and "Cryptic" splice sites in Gcc cDNA. Sequences of (a) unmodified Gcc cDNA contained in pINEX5'Gcc3' (b) In a preferred embodiment, this sequence represents modified Gcc cDNA contained in pINEX-WEIRD. The translated amino acid sequence for either the modified or unmodified Gcc cDNAs is also given, note that the modified nucleotides had no effect on the amino acid sequence.

Figure 4. (a) The sequences of the aberrantly processed transcript from the unmodified Gcc cDNA insert in pINEX5'Gcc3' and its translated polypeptide (b) Modified DNA and its translated polypeptide. In a preferred embodiment, this sequence represents modified Gcc cDNA.

DETAILED DESCRIPTION OF THE INVENTION

The invention satisfies the need for a DNA (preferably a cDNA) that when inserted into any mammalian expression vector transcribes RNA that is resistant to aberrant processing in

the transfected or transduced target cells and thus, is much more likely to translate the functional full length Gcc protein. Therefore, such a modified insert would improve the levels of Gcc expression when used in any vectors designed for *in vivo* or *ex vivo* gene therapy treatments of Gaucher disease. As well, when inserted into any efficient mammalian expression vector, such as pINEX2.0, the modified Gcc cDNA as compared to the unmodified cDNA will increase the production levels of recombinate Gcc polypeptide for use in enzyme replacement therapy for Gaucher disease. Thus, the modified insert directs a higher level of Gcc expression through a mechanism that is independent of the mammalian expression vector used whether *in vivo* or *in vitro*. The modified insert is safe as preferably no change in the amino acid sequence of Gcc is encoded by the nucleotide changes, and should confer a sustained and appropriate level of cell-specific expression for gene therapy when coupled with the appropriate vector and transfection or transduction methodologies. The expressed DNA insert is preferably a modified Gcc cDNA or a modified fragment of a Gcc cDNA that express a polypeptide having Gcc activity which is effective for treatment of Gaucher disease. The DNA is modified to prevent aberrant cellular splicing of its mRNA produced when expressed in mammalian cells. The modified DNA insert may be used with any expression vector to transfect or transduce any mammalian cell type, such as CHO cells for the expression of human Gcc for enzyme replacement. These would also include human stem cells for *ex vivo* gene therapy or macrophages for *in vivo* gene therapy.

The invention also includes the methods of making the modified DNA. The methods may be applied to a Gcc DNA from any source that requires modification to avoid undesirable splicing including humans, other mammals or synthetic DNA.

During the search to improve the efficiency of human Gcc expression it was determined that a major amount of the RNA transcribed from any vector was aberrantly spliced due to cryptic 5' and 3' splice sites contained in the human Gcc cDNA (Fig. 1 & 2). Since this RNA species encodes only a 19 amino acid peptide, it is far less stable than the properly spliced product encoding the complete 536 residues of Gcc (Maquat 1996), and therefore transcribed at a much higher level than is indicated from our steady-state RT-PCR data (Fig. 1). We modified the two cryptic sites in a manner that conserved the wild type amino acid sequence while destroying the consensus nucleotide sequences needed to induce splicing (Fig. 3). Transient expression of this modified insert indicated a 5-fold increase in Gcc expression. Such an increase in expression efficiency is not only valuable for any gene therapy approach, but also

useful in decreasing the cost of enzyme replacement since the enzyme source is now Gcc-transfected mammalian cells.

Treatment using any vector containing a modified insert to prevent aberrent transcript processing (by gene therapy or by administration of polypeptide produced from a vector) will lower the cost of the present enzyme replacement therapy (currently as much as about US$100,000 per yr. for a patient) by increasing the yield of functional Gcc protein.

The modified insert when used with any appropriate expression vector is also used to direct the expression of Gcc for use in research and characterization of the enzyme's function.

Other useful DNA inserts include a nucleic acid molecule having at least about: 50%, 60%, 70%, 80%, 90%, 95%, 99% or 99.5% sequence identity to the modified Gcc nucleic acid molecule (the Gcc sequence in figure 4(b)) wherein the molecule having sequence identity has a modification in at least one nucleotide (preferably two nucleotides) that disrupts a splicing consensus sequence and prevents splicing of mRNA while it encodes a polypeptide having Gcc activity. Changes in the Gcc nucleotide sequence which result in production of a chemically equivalent (for example, as a result of redundancy of the genetic code) or chemically similar amino acid (for example where sequence similarity is present), may also be made to produce high levels of unspliced transcript from the Gcc cDNA for therapeutic use. The DNA molecule or DNA molecule fragment may be isolated from a native source (in sense or antisense orientations) and modified or synthesized (with or without subsequent modification). It may be a mutated native or synthetic sequence or a combination of these in order to prevent or decrease aberrently spliced transcripts.

Selection of Vector

Separating the Gcc activity derived from transfected human cDNA from the endogenous Gcc activity of the host cells, e.g. CHO, was done to determine the efficiency of expression vectors. A high level of expression is needed not only for any *in vivo* or *ex vivo* gene therapy approach, but also for the efficiency of producing Gcc for enzyme replacement therapy now being done in transfected cells (Grabowski et al. 1995). We have developed an immuno-precipitation assay that is specific for the human enzyme and have used it to evaluate several expression vectors. The vector producing the highest level of Gcc in transiently transfected CHO cells was pINEX2.0 from INEX Pharmaceuticals. The vector contains a CMV-based promoter and a potential intron prior to the initiating ATG of the Gcc cDNA. In general our results indicated that a CMV-based promoter gave the highest level of expression and that the

placement of the vector's intron at the 5' end of th insert was supperior to placing it at the 3' end. Other suitable vectors will be apparent to a skilled person.

After some initial modifications to the 5' untranslated end of the cDNA construct to ensure a match with the consensus sequences for protein initiation (Kozak 1987) and the 3' end to eliminate most of the untranslated nucleotides prior to the vector's polyadenylation signal, a lysate from transiently transfected CHO cells still produced low levels of Gcc specific activity, requiring our immunoprecipitation assay to detect the increase in human activity in the cells' total Gcc pool. A line of permanently-transfected CHO cells was prepared in order to analyzed the sequence(s) of the Gcc mRNA(s) being transcribed from the expression vector.

IDENTIFICATION AND CHARACTERIZATION OF DIFFERENTIALLY SPLICED RNA

Cells Expressing Differentially Spliced RNA

A CHO cell line was permanently co-transfected with the pINEX-5'-GCC-3' construct and a construct containing a selectable marker, pREP10. After selection and isolation of individual clones, the clones were assayed to determine specific activity of Gcc (in nmole/hr/mg total lysate protein). One clone, termed A7, was grown in larger scale and RNA isolated from it. A reverse transcription reaction followed by PCR, RT-PCR, was performed on total cellular RNA from CHO control cells and A7 clone cells. Following agarose gel electrophoresis, two major bands were observed (see Fig. 1).

Restriction Digest Analysis

The major bands of the RT-PCR reaction were electrophoretically separated on a larger scale and the band(s) excised. The purified cDNA was ligated into the pCR2.1 cloning vector. Restriction digest analysis of the clones obtained using Stu I and Eco RI, revealed a series of different patterns, consistent with possible aberrant splicing (data not shown).

Sequencing Analysis

Sequencing of representative clones of each pattern, obtained from the high- and/or low-molecular weight bands, revealed a number of differentially spliced products. In the high-molecular weight band, 5 out of 10 clones contained product that was spliced at the upstream site in the vector only, producing wild-type message. One out of 10 contained a completely unspliced product, and another 1 out of 10 contained a product with a restriction map consistent with both upstream (vector) and downstream (insert) splice events taking place (Fig. 2). Remaining clones contained unidentifiable restriction maps and were therefore not sequenced.

Sequencing and restriction digest analysis of the low-molecular weight band revealed that in 7 of 10 clones both splices had taken place, and in 2 out of 10 only the upstream splice event (wild-type message) had occurred.

Sequencing results confirmed that the major alternatively spliced species resulted from the removal of sequences within the Gcc cDNA itself, through the recognition of cryptic 5' and 3' splice sites roughly corresponding to the known consensus sequences that induce RNA splicing in mammalian cells (Krawczak et al. 1992). The deduced amino acid sequence from this RNA species predicts a reading frame shift after Arg^{17} and an early stop two codons later (Fig. 3). This would encode only a 19 amino acid peptide lacking even a complete signal sequence, necessary for targeting the protein to the cell's endoplasmic reticulum. In order to eliminate aberrant splicing, the Gcc cDNA was modified by site-directed mutagenesis to alter some of the critical nucleotides making up the consensus sequences (Krawczak et al. 1992) to ensure that the cryptic splice sites no longer be recognized by the RNA processing mechanism. Care was taken to preserve the amino acid coding sequence. Figure 3 shows the consensus sequence for the 5' or 3' splice junctions (Krawczak et al. 1992), the original nucleotide sequence of the Gcc cDNA, the deduced amino acid sequence, and the modifications undertaken to destroy the consensus splicing sequences. Other modifications to destroy the consensus splicing sequences will be apparent.

Transfection Experiments

Eighteen independent transient transfection experiments were performed to compare Gcc expression levels between pINEX-5'-GCC-3' to pINEX-WEIRD. After correcting for transfection efficiency with ß-galactosidase (see Methods), pINEX-WEIRD produced 5±1 (standard error) fold higher levels of Gcc activity (see example in Table 2).

Future work will confirm that all aberrent processing of the Gcc transcript from the modified Gcc cDNA is eliminated. If not, other modifications based on the known consensus splice-sites sequences will be undertaken.

Modified DNA/DNA Having Sequence Identity

Many modifications may be made to the vector and Gcc DNA sequences and these will be apparent to one skilled in the art. The invention includes nucleotide modifications of the sequences disclosed in this application (or fragments thereof) that are capable of expressing Gcc in *in vivo* or *in vitro* cells. For example, the regulatory sequences may be modified or a nucleic acid sequence to be expressed may be modified using techniques known in the art.

Modifications include substitution, insertion or deletion of nucleotid s or altering the relativ positions or order of nucleotides. The invention includes DNA which has a sequenc with sufficient identity to a nucleotide sequence described in this application to hybridize under moderate to high stringency hybridization conditions. Hybridization techniques are well known in the art (see Sambrook et al. Molecular Cloning: A Laboratory Manual, Most Recent Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). High stringency washes have low salt (preferably about 0.2% SSC), and low stringency washes have high salt (preferably about 2% SSC). A temperature of about 37°C or about 42°C is considered low stringency, and a temperature of about 50-65°C is high stringency. The modified inserts encoding Gcc of the invention also include DNA molecules (or a fragment thereof) having at least 50% identity, at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity or, most preferred, at least 99%, 99.5% or 99.8% identity to a modified Gcc nucleic acid molecule acid as shown in figure 4(a), which have a modified consensus sequence to prevent splicing and which are capable of expressing DNA molecules *in vivo* or *in vitro*. Identity refers to the similarity of two nucleotide sequences that are aligned so that the highest order match is obtained. Identity is calculated according to methods known in the art. For example, if a nucleotide sequence (called "Sequence A") has 90% identity to the Gcc sequence in figure 4(b)], then Sequence A will be identical to the referenced portion of figure 4(b) except that Sequence A may include up to 10 point mutations (such as deletions or substitutions with other nucleotides) per each 100 nucleotides of the referenced portion of figure 4(b). The invention also includes DNA sequences which are complementary to the aforementioned sequences. "Sequence identity" may be determined, for example, by the Gap program. The algorithm of Needleman and Wunsch (1970 J Mol. Biol. 48:443-453) is used in the Gap program.

The DNA has a modification in at least one nucleotide that disrupts a splicing consensus sequence and prevents splicing of mRNA while it encodes a polypeptide having Gcc activity. This means an enzyme that can both convert the natural substrate, glucocerebroside (D-glucosylceramide), to ceramide and glucose under the appropriate conditions, and also hydrolyzed an artificial substrate, 4-methylumbelliferyl-ß-D-glucopyranoside, at a rate of greater than 10 µmoles/hr/mg of purified Gcc polypeptide.

Functionally Equivalent Nucleic Acid Molecules Identified by Hybridization

Other functionally equivalent forms of the modified Gcc DNA of the invention can be identified using conventional DNA-DNA or DNA-RNA hybridization techniques. Thus,

the present invention also includes nucleotide sequences that hybridize to the sequence in figure 4(b) or its complementary sequence, wherein the molecule that hybridizes to the Gcc portion in 4(b) has a modification in at least one nucleotide (more preferably at least two nucleotides) that disrupts a splicing consensus sequence and prevents aberrant splicing of mRNA while it encodes a polypeptide having Gcc activity. Such nucleic acid molecules preferably hybridize to the Gcc sequence in Figure 4(b) under moderate to high stringency conditions For example, high stringency washes have low salt (preferably about 0.2% SSC), and low stringency washes have high salt (preferably about 2% SSC). A temperature of about 37°C or about 42°C is considered low stringency, and a temperature of about 50-65°C is high stringency (see Sambrook et al. Molecular Cloning: A Laboratory Manual, Most Recent Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

A nucleic acid molecule is considered to be functionally equivalent to the modified Gcc nucleic acid molecules of the present invention if the nucleic acid molecule has a modification in at least one nucleotide that disrupts a splicing consensus sequence and prevents splicing of mRNA while it encodes a polypeptide having Gcc activity (Gcc activity means an enzyme that can both convert the natural substrate, glucocerebroside (D-glucosylceramide), to ceramide and glucose under the appropriate conditions, and also hydrolyzed an artificial substrate, 4-methylumbelliferyl-ß-D-glucopyranoside, at a rate of greater than 10 µmoles/hr/mg of purified Gcc polypeptide.).

Cells Containing a Vector of the Invention

The invention relates to a host cell (isolated cell *in vitro* or a cell *in vivo,* or a cell treated *ex vivo* and returned to an *in vivo* site) containing a vector and modified Gcc sequence of the invention. The preparation of transformed cells is done according to known techniques (see Materials and Methods for example of CHO cells containing a vector). The invention includes methods of expressing Gcc in the cell.

Pharmaceutical Compositions

The pharmaceutical compositions of this invention used to treat patients having Gaucher Disease could include an acceptable carrier, auxiliary or excipient. Polypeptides may be administered in pharmaceutical compositions in enzyme replacement therapy or in gene therapy.

The pharmaceutical compositions can be administered by *ex vivo* and *in vivo* methods such as electroporation, DNA microinjection, liposome DNA delivery, and virus vectors that have RNA or

DNA genomes including retrovirus vectors, lentivirus vectors, Adenovirus vectors and Adeno-associated virus (AAV) vectors. Dosages to be administered depend on patient needs, on the desired effect and on the chos n route of administration. The vectors may b introduced into the cells or their precursors using *in vivo* deliv ry vehicles such as liposomes or DNA or RNA virus vectors. They may also be introduced into these cells using physical techniques such as microinjection or chemical methods such as coprecipitation. The vector may be introduced into any mammalian cell type, such as CHO cells or human cells.

The pharmaceutical compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the vector or polypeptide is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA).

On this basis, the pharmaceutical compositions could include an active compound or substance, such as a polypeptide, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and isoosmotic with the physiological fluids. The methods of combining the vectors with the vehicles or combining them with diluents is well known to those skilled in the art. The composition could include a targeting agent for the transport of the active compound to specified sites within the mammalian cells.

Method of Medical Treatment of Gaucher Disease

Any vectors containing the DNA molecules of the invention may be administered to mammals, preferably humans, in gene therapy using techniques described below. The polypeptide produced from the modified inserts may also be administered to mammals, preferably humans, in enzyme replacement therapy.

Gene Therapy

Gene therapy to replace Gcc expression (Nolta et al. 1992; Tsai et al. 1992; Sidransky et al. 1993; Schuening et al. 1997; Dunbar et al. 1998) may be useful to modify the development or progression of Gaucher disease. The invention includes methods for providing gene therapy for treatment of diseases, disorders or abnormal physical states characterized by insufficient Gcc expression or inadequate levels or activity of Gcc polypeptide.

The invention includes methods and compositions for providing a nucleotide sequence encoding Gcc or biologically functional equivalent nucleotide sequence to the cells of an individual such that expression of Gcc in the cells provides the biological activity or phenotype of

Gcc polypeptide to those cells. Sufficient amounts of the nucleotide sequence are administered and expressed at sufficient levels to provide the biological activity or phenotype of Gcc polypeptide to the cells. For example, the method can involve a method of delivering a gene encoding Gcc to the cells of an individual having a disease, disorder or abnormal physical state, comprising administering to the individual a vector comprising DNA encoding Gcc wherein the DNA has modified sites to prevent undesirable splicing. The method may also relate to a method for providing an individual having a disease, disorder or abnormal physical state with biologically active Gcc polypeptide by administering DNA encoding Gcc. The method may be performed *ex vivo* or *in vivo*. Gene therapy methods and compositions are demonstrated, for example, in U.S. Patent Nos. 5,672,344, 5,645,829, 5,741,486, 5,656,465, 5,547,932, 5,529,774, 5,436,146, 5,399,346 and 5,670,488, 5,240,846.

The method also relates to a method for producing a stock of recombinant virus by producing virus suitable for gene therapy comprising modified DNA encoding Gcc. This method preferably involves transfecting cells permissive for virus replication (the virus containing modified Gcc) and collecting the virus produced.

Typically, a male or female is treated with the vector containing the invention (subject age will typically range from 1 to 60 years of age). At the time of treatment, he typically will have bone involvement, bone thinning and bone pain and will have an enlarged spleen and liver. The vector containing the invention is administered intravenously in order to achieve a desired level of enzyme in the patient. Treatments are repeated as deemed appropriate by a physician to ameliorate the clinical symptoms of Gaucher disease. Such treatments may be life-long.

Patients report significant improvement in bone involvement, pain and thinning, with reduction in frequency and/or intensity of pain episodes, or complete disappearance of skeletal pain often within the first six months of treatment. Patients also show improvement in cortical bone thickness. Enlargement of the spleen and liver are reduced. One of the disease markers, the enzyme chitotriosidase, shows a dramatic reduction during the course of a year.

Administration of Gcc Polypeptide

The Gcc polypeptide is administered in pharmaceutical compositions in enzyme replacement therapy, examples of which are described above (Beutler et al. 1991; Barton et al. 1992; Fallet et al. 1992; Brady et al. 1994; Grabowski et al. 1995; Rosenthal et al. 1995).

Typically, a male or female is treated with the polypeptide of the invention (subject age will typically range from 1 to 60 years of age). At the time of treatment, he typically will have bone involvement, bone thinning and bone pain and may have an enlarged spleen and liver. The polypeptide of the invention is administered intravenously at about 30U/kg every 2 weeks in order to achieve a desired level of enzyme in the patient.

Patients report significant improvement in bone involvement, pain and thinning, with reduction in frequency and/or intensity of pain episodes, or complete disappearance of skeletal pain often within the first six months of treatment. Patients also show improvement in cortical bone thickness. Enlargement of the spleen and liver are reduced. One of the disease markers, the enzyme chitotriosidase, shows a dramatic reduction during the course of a year.

Research Tool

Mammals and cell cultures transfected or transduced with vectors containing the invention are useful as research tools. Mammals and cell cultures are used in research according to numerous techniques known in the art. For example, one may obtain cells or mice (Tybulewicz et al. 1992) that express low levels of the normal or mutant Gcc polypeptide and use them in experiments to assess expression of a recombinant Gcc nucleotide sequence. In an example of such a procedure, experimental groups of mice are transformed with vectors containing recombinant Gcc genes to assess the levels of polypeptide produced, its functionality and the phenotype of the cells or mice (for example, physical characteristics of the cell structure). Some of the changes described above to optimize expression may be omitted if a lower level of expression is desired. It would be obvious to one skilled in the art that changes could be made to alter the levels of polypeptide expression.

In another example, a cell line (either an immortalized cell culture or a stem cell culture) is transformed with a DNA molecule of the invention (or variants) to measure levels of expression of the DNA molecule and the activity of the DNA molecule. For example, one may obtain mouse or human cell lines or cultures bearing the vector of the invention and obtain expression after the transfer of the cells into immunocompromised mice.

Using exogenous agents in combination with the hybrid gene

Cells transfected or transduced with a DNA molecule or polypeptide according to the invention may, in appropriate circumstances, be treated with conventional medical treatment of Gaucher disease, such as enzyme replacement therapy. The appropriate combination of treatments would be apparent to a skilled physician.

Material and Meth ds

Reagents:

All reagents used during the course of these experiments w r of research grade or molecular biology grades, as appropriate. Substrate for the acid ß-glucosidase (Gcc) activity, 4-methylumbelliferyl-ß-D-glucopyranoside (MUGc), was purchased from Sigma and purified additionally as described below. Oligonucleotide primers were obtained, as a lyophilized powder, from the Hospital for Sick Children Biotechnology Service Centre's DNA Synthesis Service. Tissue culture media (alpha-MEM) was obtained from the University of Toronto Media Preparation Service. Fetal bovine serum were obtained from CanSera through the Hospital for Sick Children Tissue Culture Service.

Lac Z, Neutral ß-Galactosidase Assay

Samples of cell lysates were diluted into water to a final volume of 60 µL. Substrate solution (190µL), prepared by dissolving 19mg of 4-MU- ß-gal (4-methylumbelliferyl- ß-galactoside) in 100 ml of pH 7.0 0.1M citrate buffer, was added. The mixture was incubated at 37°C for 30 minutes and then stopped by the addition of 2.0ml of 0.1 M MAP. Fluorescence of standard quantities of free 4-MU in 0.1 MAP (2-methyl-2-amino-1-propanol) and the assay mixtures were determined on a fluorescence spectrophotometer using 365nm excitation and 450nm emission wavelengths. Polypeptide concentration of the cell lysates were determined by the Bio-Rad method. Specific activity of the lysates were determined as nmole MU/ mg polypeptide.

Acid ß-Glucosidase (Gcc) Activity (Specific or Total):

Samples were prepared by freeze-thaw lysis (5x) in PBS containing 0.1% sodium taurocholate (NaTC), usually 100µL for a P100 dish of confluent CHO cells. A sample of the lysate (5-20µL) was diluted with 0.25% BSA to a total volume of 100µL. Reagents were added in the following order: citrate/phosphate buffer (1M/ 2M, pH 4.5), 25µL; 2% NaTC in ddH_2O, 25µL; and 20mM of the MUGc substratesolution, 100µL. The reaction was typically allowed to proceed for 1 hour at 37°C and then stopped by the addition of 3.0ml of 0.1M MAP, pH 10.5. Fluorescence of the released 4-MU was measured with the use of on a Perkin Elmer LS 30 Luminescence Spectrometer with sipper attachment. Polypeptide content was determined using the BioRad Protein Assay reagent.

Substrate solution (20mM) was prepared by dissolving MUGc in ddH_2O and heating to 40-50°C for 15-20 minutes with occasional agitation. The solution was cooled and then

extracted 3X with an equal volume of ethyl acetate. The final aqueous solution was bubbled with N_2 gas (to remove residual ethyl acetat) and aliquoted into tubes which were then frozen and stored at -20°C until needed. The substrate solution was thawed for use in a beaker of warm water, then vortexed vigorously to ensure complete dissolution of any solid material.

Immunoprecipitation Assay:

For each immunoprecipitation assay, 125µL of Goat anti-rabbit IgG coated magnetic beads (hereafter called "beads") were isolated from suspension using a permanent magnet stand (Advanced Magnetics). Beads were washed 3 times with phosphate-buffered saline containing 0.05% bovine serum albumin (PBS/BSA) by resuspension, and removal from suspension using a magnetic stand, followed by removal of the supernatant. After the final wash the beads were resuspended in 100µL of PBS/BSA and an appropriate amount of the rabbit anti-Gcc IgG (#5470), usually 4µg per assay, was added. The mixture was placed in an appropriately sized tube, depending on volume, and allowed to incubate with rotation for 4 hours at 4°C. The bead-antibody complex was precipitated with the permanent magnet stand and washed 3 times with PBS/BSA to remove any remaining free antibody and finally resuspended in 100µL of PBS/BSA. Cell lysates were prepared as above and diluted into a minimum of 400µL (to allow for adequate mixing). The washed antibody-bead complex (100µL) was added to the diluted sample and allowed to incubate overnight at 4°C with rotation. The samples were placed on ice in the permanent magnet stand and allowed to precipitate for ~30 minutes. The beads in each sample were washed (750µL) with PBS/BSA containing 0.1% Triton X-100, then twice with PBS/BSA containing 0.2% NaTC. After the final wash, the beads were resuspended in PBS/BSA/Triton and assayed for Gcc activity (as described above).

Expression of Gcc in Transiently Transfected CHO Cells

CHO cells were co-transfected with either 8µg of pINEX-5'-GCC-3' or pINEX-WEIRD and 2µg pCMV-Lac Z (encoding E. coli ß-galactosidase as a control for transfection efficiency) using Superfect Reagent (QIAGEN GmBH, Germany), according to the manufacturer's protocol. Cells were harvested after 2 days and the lysates analyzed for Gcc (using the immunoprecipitation assay) and ß-galactosidase activity. Final Gcc levels were adjusted based on the relative levels of ß-galactosidase activity in each lysate sample.

Cloned CHO Cells Permanently-Transfected with pINEX-5'-GCC-3':

CHO cells were co-transfected with 8µg of pINEX-5'-GCC-3' and 2µg pREP10 (containing a hygromycin resistence gene). Selective medium, containing 200µg/mL

hygromycin was added, and the cells were allowed to grow for approximately two weeks, splitting as necessary. After two we ks the cells were harvested by trysinization, counted using a hemocytometer and diluted as necessary to isolate single cells using 96-well dishes. After 10 days, clones that were growing well were transferred into 100mm dishes and allowed to grow for a further 10 days, splitting as necessary. Cells from each clone were harvested and assayed for Gcc activity. The final clone selected, termed A7, had the highest Gcc activity of all the clones examined.

RNA Isolation and Reverse Transcription and PCR (RT-PCR):

Cells were grown in large dishes (P150), and RNA was isolated from control CHO cells and the A7 clone, according to the one-step guanidinium isothiocyanate procedure(Chomczynski and Sacchi 1987). RNA (1µg), primer (SPR2 (see Table 1), 200pmol), RNase inhibitor, and ddH_2O (to 12.5µL total), were mixed and incubated at 65°C for 20 minutes. After cooling on ice for 5 minutes, the remaining components of the RT reaction cocktail were added (RT buffer, DTT, dNTPs, RNase inhibitor, and reverse transcriptase). The reaction cocktail (total 25µL) was incubated at 37°C for 90 min.

PCR was performed using the RT reaction products (1µL) as template. After addition of ddH_20, and primers (SPF and 53GCC2000R (see Table 1), 20pmol each), the reaction was incubated at 95°C for 5 minutes to inactivate the reverse transcriptase. The remaining reaction components (dNTPs, MgCl2, and Taq polymerase (Gibco BRL)) were used at manufacturers suggested levels. Thermocycling was performed under the following conditions: 94°C/3min; 30 cycles of 94°/1.5min, 55°/1min, 72°/1.5min; 72°/10min. Samples of the PCR reaction (10µL) were loaded onto a 1.5% agarose gel using Tris-Acetate-EDTA buffer (TAE, 40mM Tris-acetate / 2mM EDTA), electrophoresed and visualized using ethidium bromide.

Cloning of RT-PCR Products:

Electrophoresis of the RT-PCR products from the A7 clone cell line showed two major bands. One full RT-PCR reaction mixture (100µL) was separated eletrophoretically on an agarose gel, and the two major product bands were excised and purified using the Qiaex II Gel Extraction Kit (QIAGEN GmBH, Germany). The fragments were cloned into the TA cloning vector (pCR2.1) according to the manufacturer's directions (Invitrogen, Carlsbad, CA). The inserts were sequenced using either ^{35}S-T7 Sequencing Kit or ^{33}P-cycle Sequencing Kit (Amersham Pharmacia Biotech, Sweden) from either the M13 forward or M13 reverse primer

location on the vector. Sequencing gels were exposed to BioMaxMR film (Kodak) overnight and subsequently read.

Site-Directed Mutagenesis (Internal "Weird" Spice Fix):

The cryptic splice site located within the Gcc cDNA was modified by site-directed mutagenesis in order to remove potential consensus splice junction sites from the Gcc cDNA. A PCR product was obtained using one oligonucleotide primer which mutagenized a number of bases in the putative 3' junction site (3'-junction (see Table 1)) and another for the putative 5'-splice junction site (5'-junction (see Table 1)). The PCR reaction contained: 1X Pfu reaction buffer (10X stock provided by manufacturer), 0.4mM dNTP, 10ng template DNA (pINEX-5'-GCC-3'), 500ng of each oligo, and 2.5U Pfu DNA Polymerase in a final volume of 50µL in the appropriate buffer.

Amplification was performed using a Robocycler 40 Temperature Cycler (Stratagene) for 30 cycles, with temperatures and times as follows: 94°C/45 sec., 59°C / 1 min. and 72°C / 1 min. 20 sec. The PCR product was used as a mega-primer in the second round of PCR. The second PCR reaction consisted of: 5µL of the above PCR reaction mixture, 1X Pfu reaction buffer (10X stock provided by manufacturer), 0.4mM dNTPs, 50 ng template (pINEX-5'-GCC-3'), 500ng upstream oligo (SPF) and 5U Pfu DNA polymerase in a final reaction volume of 100µL. Reaction temperature conditions used were the same as for the initial PCR above. The PCR products were digested with 10U of Dra III and Xho I for 3 hr at 37°C. The plasmid pINEX-5'-GCC-3' was digested in parallel using the same method. Digested products were electrophoretically separated on an agarose gel, and the appropriate pieces were excised and purified as described above. Ligation was performed in a 20µL final volume using 5U of T4 DNA Ligase (MBI Fermentas, Lithuania), incubating overnight at 16°C to produce pINEX-WEIRD. DNA was transformed into DH5 E. coli cells (Gibco BRL) and plated onto appropriate LB agar plates containing antibiotics. Plasmid DNA was isolated and screened by restriction digest and sequencing to confirm that they contained the appropriate insert.

The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope of the invention.

All publications, patents and patent applications are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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TABLE 1: Sequ nce of Oligos Used in this Study:

Oligo Nam	Olig S qu nc * (5' to 3')
SPR2	GCCAGTGTGATGGATATCTGC
SPF	GACCGATCCAGCCTCCGGACTCT
53GCC2000R	GCCGCACACTCTGCTCCCAGAA
3-junction	CATCCGTCGCCCACTGCGTGTACTCTCATAGCGGGAAAATGT_ CAGGGCAGG
5'junction	CCTTTGAGTAGAGTCTCCATCATGGCTGGC

= Bases underlined indicate bases changed in site-directed mutagenesis PCR procedures.

TABLE 2: One of 18 Transient Expression Experiment Comparing the Wild-Type Gcc cDNA (pInex5'3'Gcc) with the Gcc cDNA Modified to Remove the Cryptic Splice Sites (pInexWEIRD)

Vector	Lac-Z (pmoles/	Corection Fact (C.F.)	Total Gcc (pmoles/hr/µg)	Human Gcc (pmoles/hr/µg)	C.F. X Hum	% pInex5'3'G
None	35	N/A	67	1.6	N/A	15
pInex5'3'Gcc	1550	1	101	10.5	8.8	100
pInexWEIRD	185	10.1	63	6.8	52.5	597